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Peptide-based compounds

Field of the invention

This invention relates to new peptide-based compounds and their use in diagnostic optical imaging techniques. More specifically the invention relates to the use of such peptide-based compounds as targeting vectors that bind to receptors associated with angiogenesis. The compounds may be used as contrast agents in diagnosis of angiogenesis-related diseases.

Background of invention

Generally, new blood vessels can be formed by two different mechanisms: vasculogenesis or angiogenesis. Angiogenesis is the formation of new blood vessels by branching from existing vessels. The primary stimulus for this process may be inadequate supply of nutrients and oxygen (hypoxia) to cells in a tissue. The cells may respond by secreting angiogenic factors, of which there are many; one example, which is frequently referred to, is vascular endothelial growth factor (VEGF). These factors initiate the secretion of proteolytic enzymes that break down the proteins of the basement membrane, as well as inhibitors that limit the action of these potentially harmful enzymes. The other prominent effect of angiogenic factors is to cause endothelial cells to migrate and divide. Endothelial cells that are attached to the basement membrane, which forms a continuous sheet around blood vessels on the contralumenal side, do not undergo mitosis. The combined effect of loss of attachment and signals from the receptors for angiogenic factors is to cause the endothelial cells to move, multiply, and rearrange themselves, and finally to synthesise a basement membrane around the new vessels.

Angiogenesis is prominent in the growth and remodelling of tissues, including wound healing and inflammatory processes. Inhibition of angiogenesis is also considered to be a promising strategy for antitumour therapy. The transformations accompanying angiogenesis are also very promising for diagnosis, an obvious example being malignant disease, but the concept also shows great promise in inflammation and a variety of inflammation-related diseases, including atherosclerosis, the macrophages of early atherosclerotic lesions being potential sources of angiogenic factors. These factors are also involved in re-vascularisation of infarcted parts of the myocardium, which occurs if a stenosis is released within a short time.

Reference is made to WO 98/47541, WO01/77145 and WO 03/006491 for further examples of undesired conditions that are associated with neovascularization or angiogenesis. Relevant conditions are arteriovenous malformations, astrocytomas, atherosclerosis,breast cancers, choriocarcinomas, colorectal cancers, gingivitis, glioblastomas, gliomas, hemangiomas (childhood, capillary), hepatomas, hyperplastic endometrium, inflammation (e.g. chronic), ischemic myocardium, Kaposi sarcoma, lung cancers, macular degeneration, melanoma, metastasis, neuroblastomas, occluding peripheral artery disease, osteoarthritis, ovarian cancers, pancreatic cancers, prostate cancers, psoriasis, retinopathy (diabetic, proliferative), rheumatoid, arthritis, scleroderma, seminomas, skin cancers, solid tumor formation, ulcerative colitis.

Angiogenesis involves receptors that are unique to endothelial cells and surrounding tissues. These markers include growth factor receptors such as VEGF and the

Integrin family of receptors. Immunohistochemical studies have demonstrated that a variety of integrins, perhaps most importantly the α_v class, are expressed on the apical surface of blood vessels [Conforti, G., et al. (1992) Blood 80: 37-446] and are available for targeting by circulating ligands [Pasqualini, R., et al. (1997) Nature Biotechnology 15: 542-546]. The α5β1 is also an important integrin in promoting the assembly of fibronectin matrix and initiating cell attachment to fibronectin. It also plays a crucial role in cell migration.

The integrin αvβ3 is one of the receptors that is known to be associated with angiogenesis. Stimulated endothelial cells appear to rely on this receptor for survival during a critical period of the angiogeneic process, as antagonists of the αvβ3 integrin receptor/ligand interaction induce apoptosis and inhibit blood vessel growth.

Integrins are heterodimeric molecules in which the α- and β-subunits penetrate the cell-membrane lipid bilayer. The α-subunit has four Ca^{2+} binding domains on its extracellular chain, and the β-subunit has a number of extracellular cysteine-rich domains.

Many ligands (eg. fibronectin) involved in cell adhesion contain the tripeptide sequence arginine-glycine-aspartic acid (RGD). The RGD sequence appears to act as a primary recognition site between the ligands presenting this sequence and receptors on the surface of cells. It is generally believed that secondary interactions between the ligand and receptor enhance the specificity of the interaction. These secondary interactions might take place between moieties of the ligand and receptor that

are immediately adjacent to the RGD sequence or at sites that are distant from the RGD sequence.

RGD peptides are known to bind to a range of integrin receptors and have the potential to regulate a number of cellular events of significant application in the clinical setting. Perhaps the most widely studied effect of RGD peptides and mimetics thereof relate to their use as anti-thrombotic agents where they target the platelet integrin GpIIbIIIa.

Inhibition of angiogenesis in tissues by administration of either an αvβ3 or αvβ5 antagonist has been described in for example WO 97/06791 and WO 95/25543 using either antibodies or RGD containing peptides. EP 578083 describes a series of mono-cyclic RGD containing peptides and WO 90/14103 claims RGD-antibodies. Haubner et al. in the J. Nucl. Med. (1999); 40: 1061-1071 describe a new class of tracers for tumour targeting based on monocyclic RGD containing peptides. Biodistribution studies using whole-body autoradiographic imaging revealed however that the ^{125}I-labelled peptides had very fast blood clearance rates and predominantly hepatobiliary excretion routes resulting in high background.

Cyclic RGD peptides containing multiple bridges have also been described in WO 98/54347 and WO 95/14714. Peptides derived from in vivo biopanning (WO 97/10507) have been used for a variety of targeting applications. The sequence CDCRGDCFC (RGD-4C), has been used to target drugs such as doxirubicin (WO 98/10795), nucleic acids and adenoviruses to cells (see WO 99/40214, WO 99/39734, WO 98/54347, WO 98/54346, US 5846782). Peptides containing multiple cysteine residues do however suffer

from the disadvantage that multiple disulphide isomers can occur. A peptide with 4 cysteine residues such as RGD-4C has the possibility of forming 3 different disulphide folded forms. The isomers will have varying affinity for the integrin receptor as the RGD pharmacophore is forced into 3 different conformations.

Further examples of RGD comprising peptide-based compounds are found in WO01/77145 and WO02/26776.

There is a clinical need to develop more specific non-invasive imaging techniques for angiogenesis-related diseases. Such imaging techniques will have a central role in the evaluation of novel anti-angiogenic therapies. Being able to assess the actual level of angiogenesis will be of clinical benefit in diagnosing angiogenesis-related diseases at an early stage. It has now surprisingly been found that optical imaging may be used to assess the level of angiogenesis, and the invention provides new optical contrast agents for this purpose.

Summary of the invention

In view of the needs of the art the present invention provides peptide-based compounds labeled with fluorescein for use as contrast agents in optical imaging. The efficient targeting and imaging of integrin receptors associated with angiogenesis in vivo demands a selective, high affinity RGD based vector that is chemically robust and stable. Furthermore, the route of excretion is an important factor when designing imaging agents in order to reduce problems with background. These stringent conditions are met by the bicyclic fluorescein-labeled peptide structures described in the present invention.

Detailed description of the invention

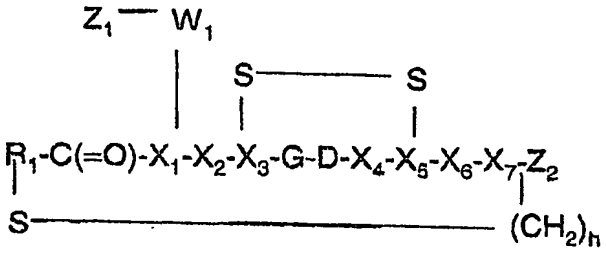
Viewed from one aspect the invention provides new peptide-based compounds of formula I as defined in the claims. These compounds have affinity for integrin receptors, e.g. affinity for the integrin αvβ3, and are labeled with the dye reporter fluorescein.

The compounds of formula I comprise at least two bridges, wherein one bridge forms a disulphide bond and the second bridge comprises a thioether (sulphide) bond and wherein the bridges fold the peptide moiety into a 'nested' configuration.

The compounds of the current invention thus have a maximum of one disulphide bridge per molecule moiety. Compounds defined by the present invention are surprisingly stable in vivo and under the conditions employed during labeling with fluorescein.

These new compounds may be used as contrast agents in optical imaging.

The peptide-based compounds described in the present invention are defined by formula (I):



(I)

or physiologically acceptable salts thereof

wherein
G represents glycine, and
D represents aspartic acid, and
R_1 represents $-(CH_2)n-$ or $-(CH_2)n-C_6H_4-$, preferably R_1 represents $-(CH_2)-$ wherein
n represents a positive integer between 1 and 10, and
h represents a positive integer 1 or 2, and

X_1 represents an amino acid residue wherein said amino acid possesses a functional side-chain such as an acid or amine, preferably aspartic or glutamic acid, lysine, homolysine diaminoalcylic acid or diaminopropionic acid,

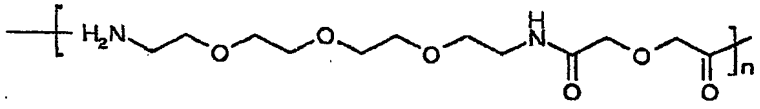
X_2 and X_4 represent independently an amino acid residue capable of forming a disulphide bond, preferably a cysteine or a homocysteine residue, and

X_3 represents arginine, N-methylarginine or an arginine mimetic, preferably an arginine, and

X_5 represents a hydrophobic amino acid or derivatives thereof, preferably a tyrosine, a phenylalanine, a 3-iodo-tyrosine or a naphthylalanine residue, and more preferably a phenylalanine or a 3-iodo-tyrosine residue, and

X_6 represents a thiol-containing amino acid residue, preferably a cysteine or a homocysteine residue, and

X_7 is absent or represents a spacer moiety or a biomodifier moiety preferably based on a monodisperse polyethylene glycol (PEG) building block comprising 1 to 10 units of said building block, said biomodifier having the function of modifying the pharmacokinetics and blood clearance rates of said agents. In addition X_7 may also represent 1 to 10 amino acid residues preferably comprising glycine, lysine, aspartic acid or serine. X_7 may also represent a spacer or biomodifier comprising both amino acid residues and a PEG-like structure, preferably a bis aminoethyl ethylene glycol glycine combination. In a preferred embodiment X_7 represents a unit comprised of the monodisperse PEG-like structure, 17-amino-5-oxo-6-aza-3,9,12,15-tetraoxaheptadecanoic acid of formula (II),



(II)

wherein n equals an integer from 1 to 10 and were the C-terminal end is an amide or acid moiety.

Z_1 and Z_2 represent fluorescein or derivatives thereof or is absent, such that the compound comprises at least one fluorescein moiety,

W_1 is absent or represents a spacer moiety and is preferentially derived from glutaric and/or succinic acid and/or a polyethylenglycol based unit linking the

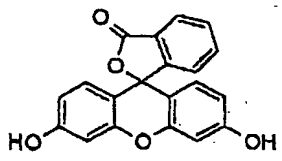
fluorescein reporter to the peptide. Other representative spacer (W_1) elements include structural-type polysaccharides, storage-type polysaccharides, polyamino acids and methyl and ethyl esters thereof, and polypeptides, oligosaccharides and oligonucleotides, which may or may not contain enzyme cleavage sites.

The role of the spacer moiety W_1 is to distance the relatively bulky fluorescein reporter from the receptor binding domain of the peptide component.

In the formula I C(=O) represents a carbonyl moiety.

It is found that the biomodifier, X_7, modifies the pharmacokinetics and blood clearance rates of the compounds. The biomodifier effects less uptake of the compounds in tissue i.e. muscle, liver etc. thus giving a better diagnostic image due to less background interference. The secretion is mainly through the kidneys due to a further advantage of the biomodifier.

The reporter Z_1 and Z_2 comprise fluorescein or derivatives of fluourescein. Formula (III) gives fluorescein.



(III)

Fluorescein, represented by Z_1 and/or Z_2, may be linked to X_7 and/or W_1 by amide bond formation with a suitable amino group of the peptide. Active esters of fluorescein such as the NHS ester (Pierce Catalog 46100) are considered particularly useful.

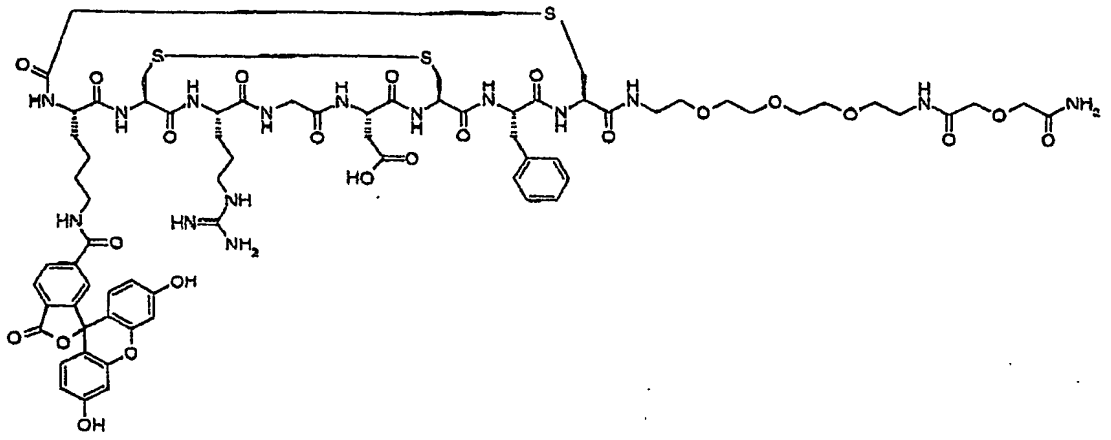
A preferred embodiment of the invention relates to an optical imaging contrast agent of formula (I), particularly for use in optical imaging of and diagnosing of angiogenesis-related diseases.

In most cases, it is preferred that the amino acids in the peptide are all in the L-form. However, in some embodiments of the invention one, two, three or more of the amino acids in the peptide are preferably in the D-form. The inclusion of such D-form amino acids can have a significant effect on the serum stability of the compound.

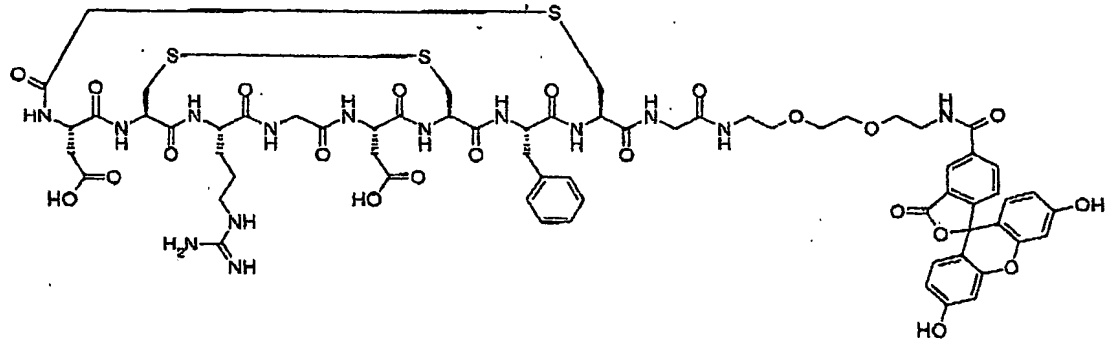
According to the present invention, any of the amino acid residues as defined in formula I may preferably represent a naturally occurring amino acid and independently in any of the D or L conformations.

Some of the compounds of the invention are high affinity RGD based vectors. As used herein the term 'high affinity RGD based vector' refers to compounds that have a Ki of < 10 nM and preferably < 5 nM, in a competitive binding assay for $\alpha v\beta 3$ integrin and where the Ki value was determined by competition with the known high affinity ligand echistatin. Methods for carrying out such competition assays are well known in the art.
Some preferred embodiments of the compounds of formula I are illustrated by the compounds below:

Compound I:



Compound II:



The present invention also provides a pharmaceutical composition comprising an effective amount, e.g. an amount effective for enhancing image contrast in in vivo imaging of a compound of general formula I or a salt thereof, together with one or more pharmaceutically acceptable adjuvants, excipients or diluents.

The invention further provides a pharmaceutical composition for treatment of a disease comprising an effective amount of a compound of general formula I, or an acid addition salt thereof, together with one or more pharmaceutically acceptable adjuvants, excipients or diluents.

Viewed from a further aspect the invention provides the use of a compound of formula I for the manufacture of an optical imaging contrast medium for use in a method of diagnosis involving administration of said contrast medium to a human or animal body and generation of an image of at least part of said body.

Use of the compounds of formula I in the manufacture of therapeutic compositions (medicament) and in methods of therapeutic or prophylactic treatment, preferably treatment of angiogenesis-related diseases, of the human or animal body are thus considered to represent further aspects of the invention.

Viewed from a still further aspect the invention provides a method of generating an image of a human or animal body by optical imaging involving administering a contrast agent to said body, e.g. into the vascular system and generating an image of at least a part of said body, to which said contrast agent has distributed, wherein as said contrast agent is used an agent of formula I.

Viewed from a still further aspect the invention provides a method of generating enhanced images of a human or animal body by optical imaging previously administered with a contrast agent composition comprising a compound as defined by formula I, which method comprises generating an image of at least part of said body.

Viewed from a further aspect the invention provides a method of monitoring the effect of treatment of a human or animal body with a drug to combat a condition associated with angiogenesis, said method involving

administering to said body an agent of formula I and detecting the uptake of said agent by cell receptors, preferably endothelial cell receptors and in particular αvβ3 receptors, said administration and detection optionally but preferably being effected repeatedly, e.g. before, during and after treatment with said drug. Said detection comprising an optical imaging technique.

Since fluorescein as well as the fluorescein-peptide conjugate of the present invention emits in the visual range of the spectrum, conventional ophthalmoscopy equipment may be used for acquiring the images. Furthermore, in-vivo confocal microscopy may be applied. Recently developed time-domain and frequency-domain imaging techniques may potentially also be used, taking advantage of additional characteristics of the fluorophore, such as lifetime.

The compounds of the present invention can be synthesized using all the known methods of chemical synthesis but particularly useful is the solid-phase methodology of Merrifield employing an automated peptide synthesizer (J. Am. Chem. Soc., 85: 2149 (1964)). In addition coupling of fluorescein, such as a fluorescein active ester, can also be carried out automatically yielding an amide bond between the peptide and fluorescein-moiety. The peptides and peptide conjugates may be purified using high performance liquid chromatography (HPLC) and characterized by mass spectrometry and analytical HPLC before testing in the in vitro screen.

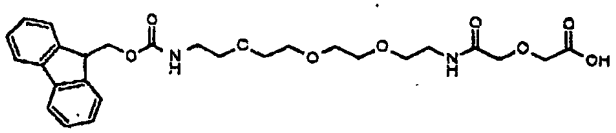
The present invention will now be further illustrated by way of the following non-limiting examples.

Examples

Example 1:

Synthesis of disulphide [Cys^{2-6}] thioether cyclo[CH_2CO-Lys(fluorescein)-Cys^2-Arg-Gly-Asp-Cys^6-Phe-Cys]-PEG-NH_2

1 a) Synthesis of 17-(Fmoc-amino)-5-oxo-6-aza-3,9,12,15-tetraoxaheptadecanoic acid



This building block is coupled to the solid-phase using Fmoc chemistry. The coupled form of this building block will be referred to in short as PEG.

1,11-Diazido-3,6,9-trioxaundecane

A solution of dry tetraethylene glycol (19.4 g, 0.100 mol) and methanesulphonyl chloride (25.2 g, 0.220 mol) in dry THF (100 ml) was kept under argon and cooled to 0 °C in an ice/water bath. To the flask was added a solution of triethylamine (22.6 g, 0.220 mol) in dry THF (25 ml) dropwise over 45 min. After 1 hr the cooling bath was removed and stirring was continued for 4 hrs. Water (60 ml) was added. To the mixture was added sodium hydrogencarbonate (6 g, to pH 8) and sodium azide (14.3 g, 0.220 mmol), in that order. THF was removed by distillation and the aqueous solution was refluxed for 24 h (two layers formed). The mixture was cooled and ether (100 ml) was added. The aqueous phase was saturated with sodium chloride. The phases were separated and the aqueous phase was extracted with ether (4 x 50 ml).

Combined organic phases were washed with brine (2 x 50 ml) and dried ($MgSO_4$). Filtration and concentration gave 22.1 g (91%) of yellow oil. The product was used in the next step without further purification.

11-Azido-3,6,9-trioxaundecanamine

To a mechanically, vigorously stirred suspension of 1,11-diazido-3,6,9-trioxaundecane (20.8 g, 0.085 mol) in 5% hydrochloric acid (200 ml) was added a solution of triphenylphosphine (19.9 g, 0.073 mol) in ether (150 ml) over 3 hrs at room temperature. The reaction mixture was stirred for additional 24 hrs. The phases were separated and the aqueous phase was extracted with dichloromethane (3 x 40 ml). The aqueous phase was cooled in an ice/water bath and pH was adjusted to ca 12 by addition of KOH. The product was extracted into dichloromethane (5 x 50 ml). Combined organic phases were dried ($MgSO_4$). Filtration and evaporation gave 14.0 g (88%) of yellow oil. Analysis by MALDI-TOF mass spectroscopy (matrix: α-cyano-4-hydroxycinnamic acid) gave a M+H peak at 219 as expected. Further characterisation using ^{1}H (500 MHz) and ^{13}C (125 MHz) NMR spectroscopy verified the structure.

17-Azido-5-oxo-6-aza-3,9,12,15-tetraoxaheptadecanoic acid

To a solution of 11-azido-3,6,9-trioxaundecanamine (10.9 g, 50.0 mmol) in dichloromethane (100 ml) was added diglycolic anhydride (6.38 g, 55.0 mmol). The reaction mixture was stirred overnight. HPLC analysis (column Vydac 218TP54; solvents: A = water/0.1% TFA and B = acetonitrile/0.1% TFA; gradient 4-16% B over 20 min; flow 1.0 ml/min; UV detection at 214 and 284 nm), showed complete conversion of starting material to a product with retention time 18.3 min. The solution was

concentrated to give quantitative yield of a yellow syrup. The product was analysed by LC-MS (ES ionisation) giving [MH]+ at 335 as expected. ^{1}H (500 MHz) and ^{13}C (125 MHz) NMR spectroscopy was in agreement with structure The product was used in the next step without further purification.

17-Amino-5-oxo-6-aza-3,9,12,15-tetraoxaheptadecanoic acid

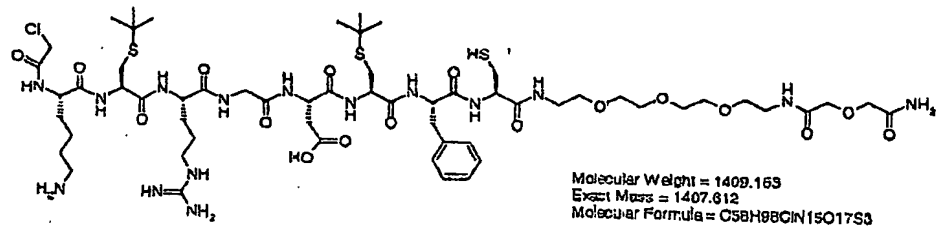
A solution of 17-azido-5-oxo-6-aza-3,9,12,15-tetraoxaheptadecanoic acid (8.36 g, 25.0 mmol) in water (100 ml) was reduced using H_2(g)-Pd/C (10%). The reaction was run until LC-MS analysis showed complete conversion of starting material (column Vydac 218TP54; solvents: A = water/0.1% TFA and B = acetonitrile/0.1% TFA; gradient 4-16% B over 20 min; flow 1.0 ml/min; UV detection at 214 and 284 nm, ES ionisation giving M+H at 335 for starting material and 309 for the product). The solution was filtered and used directly in the next step.

17-(Fmoc-amino)-5-oxo-6-aza-3,9,12,15-tetraoxaheptadecanoic acid

To the aqueous solution of 17-amino-5-oxo-6-aza-3,9,12,15-tetraoxaheptadecanoic acid from above(corresponding to 25.0 mmol amino acid) was added sodium bicarbonate (5.04 g, 60.0 mmol) and dioxan (40 ml). A solution of Fmoc-chloride (7.11 g, 0.275 mol) in dioxan (40 ml) was added dropwise. The reaction mixture was stirred overnight. Dioxan was evaporated off (rotavapor) and the aqueous phase was extracted with ethyl acetate. The aqueous phase was acidified by addition of hydrochloric acid and precipitated material was extracted into chloroform. The organic phase was dried ($MgSO_4$), filtered and concentrated to give 11.3 g

(85%) of a yellow syrup. The structure was confirmed by LC-MS analysis (column Vydac 218TP54; solvents: A = water/0.1% TFA and B = acetonitrile/0.1% TFA; gradient 40-60% B over 20 min; flow 1.0 ml/min; UV detection at 214 and 254 nm, ES ionisation giving M+H at 531 as expected for the product peak at 5,8 minutes). The analysis showed very low content of side products and the material was used without further purification.

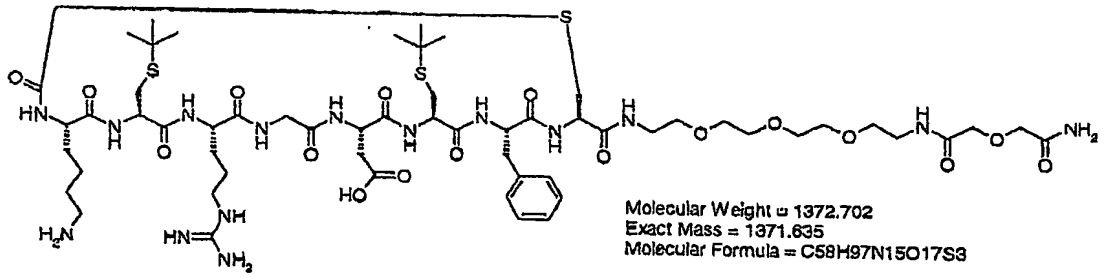
1 b) Synthesis of $ClCH_2CO$-Lys-Cys(tBu)-Arg-Gly-Asp-Cys(tBu)-Phe-Cys-PEG-NH_2



The PEG unit was coupled manually to Rink Amide AM resin, starting on a 0.25 mmol scale, mediated by HATU activation. The remaining peptide was assembled on an ABI 433A automatic peptide synthesiser using 1 mmol amino acid cartridges. The amino acids were pre-activated using HBTU before coupling. N-terminal amine groups were chloroacetylated using a solution of chloroacetic anhydride in DMF for 30 min. The simultaneous removal of peptide and side-chain protecting groups (except tBu) from the resin was carried out in TFA containing TIS (5 %), H_2O (5 %) and phenol (2.5 %) for two hours. After work-up 322 mg of crude peptide was obtained (Analytical HPLC: Gradient, 5-50 % B over 10 min where A = H_2O/0.1 % TFA and B = CH_3CN/0.1 % TFA; column, Phenomenex Luna 3 µ C18 (2) 50 x 4.6 mm; flow, 2 mL/min; detection, UV 214 nm; product retention time, 6.37 min). Further product

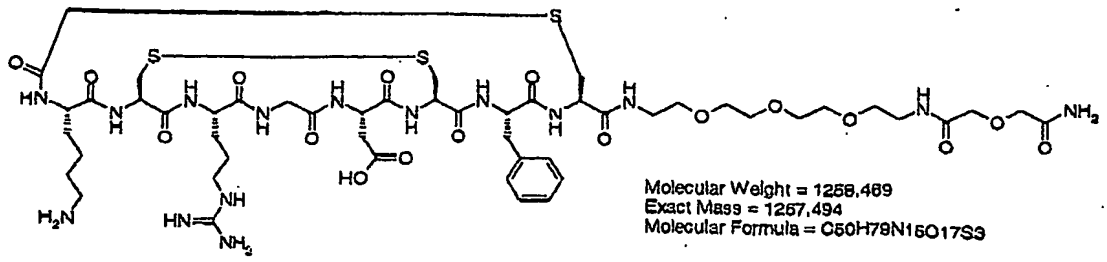
characterisation was carried out using mass spectrometry: Expected, M+H at 1409, found, at 1415).

1 c) Synthesis of thioether cyclo[CH_2CO-Lys-Cys(tBu)-Arg-Gly-Asp-Cys(tBu)-Phe-Cys]-PEG-NH_2



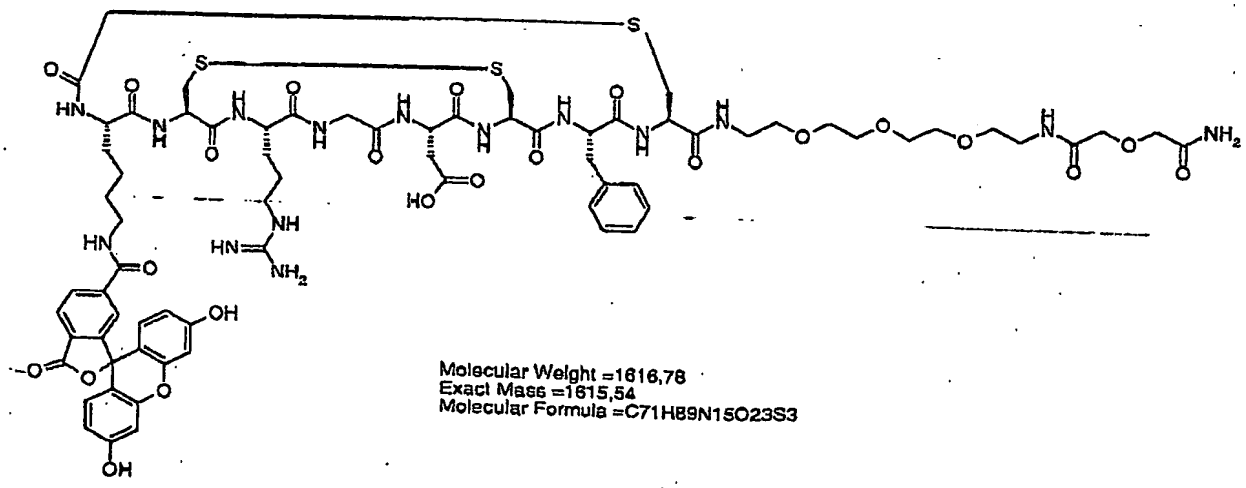
322 mg of $ClCH_2CO$-Lys-Cys(tBu)-Arg-Gly-Asp-Cys(tBu)-Phe-Cys-(PEG)n-NH_2 was dissolved in water/acetonitrile. The mixture was adjusted to pH 8 with ammonia solution and stirred for 16 hours. After work-up crude peptide was obtained (Analytical HPLC: Gradient, 5-50 % B over 10 min where A = H_2O/0.1 % TFA and B = CH_3CN/0.1 % TFA; column, Phenomenex Luna 3 µ C18 (2) 50 x 4.6 mm; flow, 2 mL/min; detection, UV 214 nm; product retention time, 6.22 min). Further product characterisation was carried out using mass spectrometry: Expected, M+H at 1373, found, at 1378).

1 d) Synthesis of disulphide [Cys^{2-6}] thioether cyclo[CH_2CO-Lys-Cys^2-Arg-Gly-Asp-Cys^6-Phe-Cys]-PEG-NH_2



Thioether cyclo[CH_2CO-Lys-Cys(tBu)-Arg-Gly-Asp-Cys(tBu)-Phe-Cys]-(PEG)n-NH_2 was treated with a solution of anisole (200 µL), DMSO (2 mL) and TFA (100 mL) for 60 min following which the TFA was removed in vacuo and the peptide precipitated by the addition of diethyl ether. Purification by preparative HPLC (Phenomenex Luna 5 µ C18 (2) 250 x 21.20 mm column) of 70 mg crude material was carried out using 0-30 % B, where A = H_2O/0.1 % TFA and B = CH_3CN/0.1 % TFA, over 40 min at a flow rate of 10 mL/min. After lyophilisation 46 mg of pure material was obtained (Analytical HPLC: Gradient, 0-30 % B over 10 min where A = H_2O/0.1 % TFA and B = CH_3CN/0.1 % TFA; column, Phenomenex Luna 3 µ C18 (2), 50 x 4.6 mm; flow, 2 mL/min; detection, UV 214 nm; product retention time, 6.80 min). Further product characterisation was carried out using mass spectrometry: Expected, M+H at 1258.5, found, at 1258.8).

1 e) Synthesis of disulfide [Cys^{2-6}] thioether cyclo[CH_2CO-Lys(fluorescein)-Cys^2-Arg-Gly-Asp-Cys^6-Phe-Cys]-PEG-NH_2

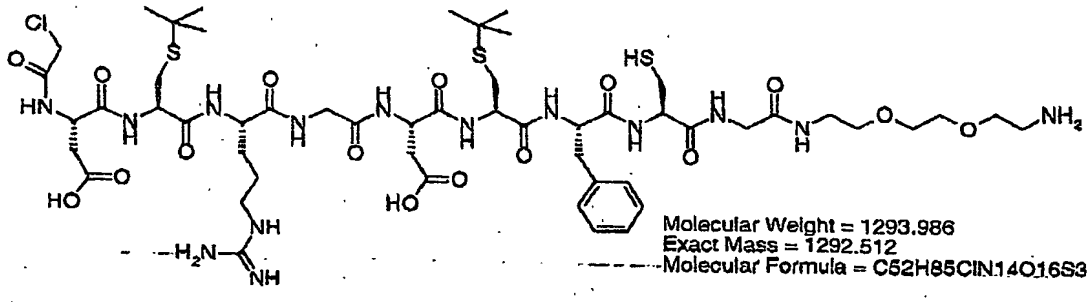


30 mg of [Cys^{2-6}] cyclo[CH_2CO-Lys-Cys-Arg-Gly-Asp-Cys-Phe-Cys]-PEG-NH_2, 16.2 mg of NHS-Fluorescein and 4 µL of N-

methylmorpholine was dissolved in DMF (3 mL). The mixture was protected against light and stirred over night. Purification by preparative HPLC (Vydac 218TP1022 C18 column) of the reaction mixture was carried out using 20-30 % B, where A = H_2O/0.1 % TFA and B = CH_3CN/0.1 % TFA, over 40 min at a flow rate of 10 mL/min. After lyophilisation 21.6 mg of pure material was obtained (Analytical HPLC: Gradient, 10-40 % B over 10 min where A = H_2O/0.1 % TFA and B = CH_3CN/0.1 % TFA; column, Phenomenex Luna 3 µ C18 (2) 50 x 4.6 mm; flow, 2 mL/min; detection, UV 214 nm; product retention time, 7.0 min). Further product characterisation was carried out using mass spectrometry: Expected, M+H at 1616.5, found, at 1616.3).

Example 2: Synthesis of disulphide [Cys $^{2-6}$]thioether cyclo[CH_2CO-Asp-Cys^2-Arg-Gly-Asp-Cys-Phe-Cys-Gly]-Bis(aminoethyl)ethylene glycol-fluorescein

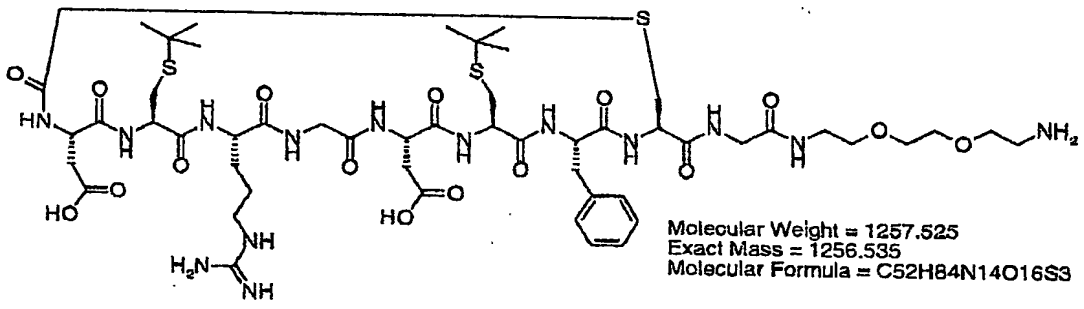
2a. Synthesis of $ClCH_2CONH$-Asp-Cys(tBu)-Arg-Gly-Asp-Cys(tBu)-Phe-Cys-Gly-NH-$(CH_2CH_2O)_2CH_2CH_2NH_2$



The peptide was synthesised on a ABI 433A automatic peptide synthesiser starting with O-Bis-(aminoethyl)ethylene glycol trityl resin on a 0.25 mmol scale using 1 mmol amino cartridges. The amino acids were pre-activated using HBTU before coupling. The N-terminal was chloroacetylated using chloroacetic anhydride. The simultaneous removal of peptide and side-chain protecting

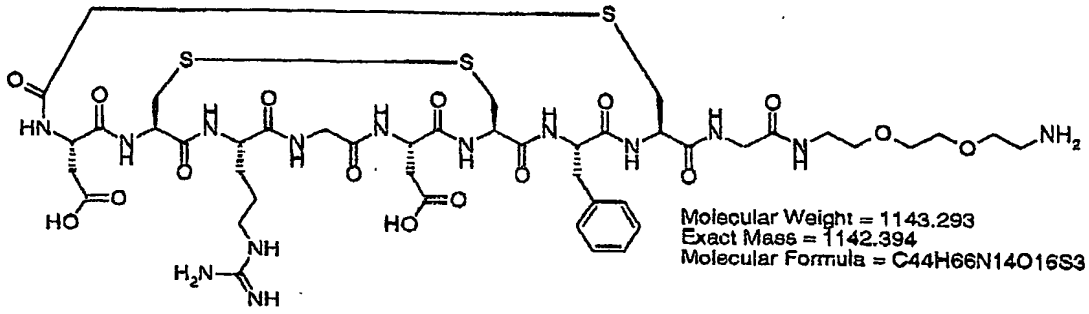
groups (except tBu) from the resin was carried out in TFA containing TIS (5 %), H_2O (5 %) and phenol (2.5 %) for two hours. After work-up 364 mg of crude peptide was obtained (Analytical HPLC: Gradient, 5-50 % B over 10 min where A = H_2O/0.1 % TFA and B = CH_3CN/0.1 % TFA; 2 mL/min; column, Phenomenex Luna 3µ C18 (2) 50 x 4.6 mm; detection, UV 214 nm; product retention time, 6.55 min). Further product characterisation was carried out using electrospray mass spectrometry: expected, M+H at 1293.5, found, at 1293.4).

2b. Synthesis of cyclo[CH_2CONH-Asp-Cys(tBu)-Arg-Gly-Asp-Cys(tBu)-Phe-Cys]-Gly-NH-$(CH_2CH_2O)_2CH_2CH_2NH_2$



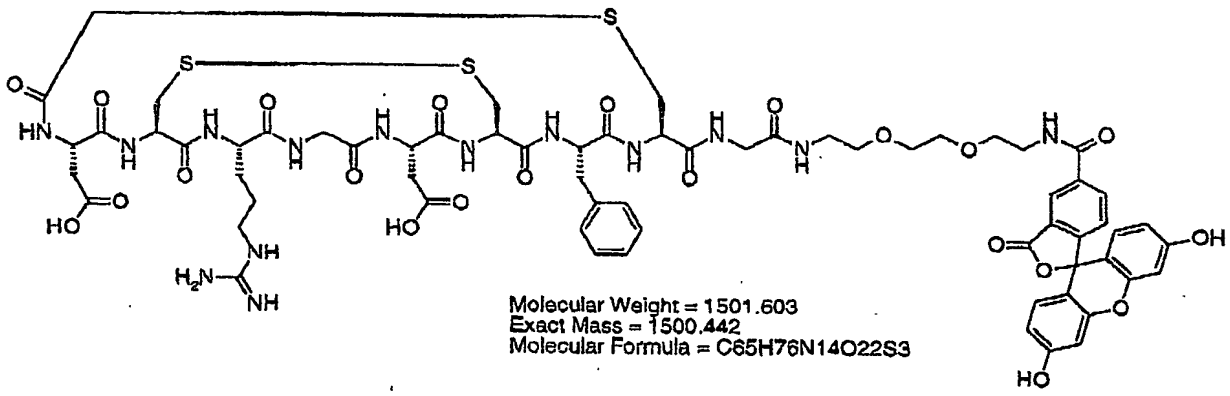
250 mg of $ClCH_2CONH$-Asp-Cys(tBu)-Arg-Gly-Asp-Cys(tBu)-Phe-Cys-Gly-NH-$(CH_2CH_2O)_2CH_2CH_2NH_2$ was dissolved in water/acetonitrile. The mixture was adjusted to pH 8 with ammonia solution and stirred for 18 hours.
After lyophilisation the crude peptide was obtained as a white powder. (Analytical HPLC: Gradient, 5-50 % B over 10 min where A = H_2O/0.1 % TFA and B = CH_3CN/0.1 % TFA; flow, 2 mL/min; column, Phenomenex Luna 3µ C18 (2) 50 x 4.6 mm; detection, UV 214 nm; product retention time, 6.17 min). Further product characterisation was carried out using electrospray mass spectrometry: expected, M+H at 1257.5, found, at 1257.6).

2c. Synthesis of [Cys$^{2-6}$] cyclo[CH_2CONH-Asp-Cys-Arg-Gly-Asp-Cys-Phe-Cys]-Gly-NH-$(CH_2CH_2O)_2CH_2CH_2NH_2$



Cyclo[CH_2CONH-Asp-Cys(tBu)-Arg-Gly-Asp-Cys(tBu)-Phe-Cys]-Gly-NH-$(CH_2CH_2O)_2CH_2CH_2NH_2$ was dissolved in a solution of anisole (500 µl), DMSO (4 ml) and TFA (200 ml). The mixture was stirred at room temperature for 15 min following which the TFA was removed *in vacuo* and the peptide precipitated by the addition of diethyl ether. Purification by preparative HPLC (Phenomenex Luna 5µ C18 (2) 250 x 21.20 mm column) of the crude material was carried out using 0-30 % B, where A = H_2O/0.1 % TFA and B = CH_3CN/0.1 % TFA, over 40 min at a flow rate of 10 mL/min. After lyophilisation 44 mg of pure material was obtained. (Analytical HPLC: Gradient, 0-30 % B over 10 min where A = H_2O/0.1 % TFA and B = CH_3CN/0.1 % TFA; flow, 2 mL/min; column, Phenomenex Luna 3µ C18 (2) 50 x 4.6 mm; detection, UV 214 nm; product retention time, 5.88 min). Further product characterization was carried out using electrospray mass spectrometry: expected, M+H at 1143.4, found, at 1143.5).

2d. Conjugation of [Cys$^{2-6}$] cyclo[CH_2CONH-Asp-Cys-Arg-Gly-Asp-Cys-Phe-Cys]-Gly-NH-$(CH_2CH_2O)_2CH_2CH_2NH_2$ with fluorescein



10 mg of [Cys$^{2-6}$] cyclo[CH_2CONH-Asp-Cys-Arg-Gly-Asp-Cys-Phe-Cys]-Gly-NH-$(CH_2CH_2O)_2CH_2CH_2NH_2$, 4.7 mg of fluorescein active ester and 5 µL of 4-methylmorpholine were dissolved in DMF (0.5 mL) and the solution stirred for 3 hours.

Purification by preparative HPLC (Phenomenex Luna 5µ C18 (2) 250 x 21.20 mm column) of the crude material was carried out using 0-30 % B, where A = H_2O/0.1 % TFA and B = CH_3CN/0.1 % TFA, over 40 min at a flow rate of 10 mL/min. After lyophilisation 6 mg of pure material was obtained. (Analytical HPLC: Gradient, 0-30 % B over 10 min where A = H_2O/0.1 % TFA and B = CH_3CN/0.1 % TFA; flow, 2 mL/min; column, Phenomenex Luna 3µ C18 (2) 50 x 4.6 mm; detection, UV 214 nm; product retention time, 10.07 min). Further product characterisation was carried out using electrospray mass spectrometry: expected, M+H at 1501.4, found, at 1501.4).

SEQUENCE LISTING

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<170> PatentIn version 3.1

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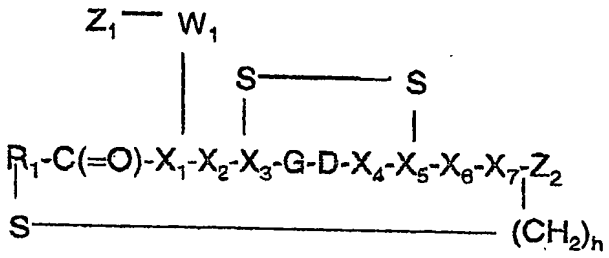
Claims:

1. A compound of general formula (I)



(I)

or a physiologically acceptable salts thereof

wherein
G represents glycine
D represents aspartic acid
R_1 represents $-(CH_2)n-$ or $-(CH_2)n-C_6H_4-$ wherein
n represents a positive integer between 1 and 10
h represents a positive integer 1 or 2, and

X_1 represents an amino acid residue wherein said amino acid possesses a functional side-chain such as an acid or amine,

X_2 and X_4 represent independently an amino acid residue capable of forming a disulphide bond,

X_3 represents arginine, N-methylarginine or an arginine mimetic,

X_5 represents a hydrophobic amino acid or derivatives thereof, and

X_6 represents a thiol-containing amino acid residue, and

X_7 is absent or represents a spacer or biomodifier moiety, and

Z_1 and Z_2 represent fluorescein, or derivatives thereof, or one Z-group is absent, such that the compound comprises at least one fluorescein moiety, and

W_1 is absent or represents a spacer moiety.

2. A compound as claimed in claim 1 wherein any of the amino acid residues are independently in the D or L conformation.

3. A compound as claimed in claim 1 wherein R_1 represents $-(CH_2)-$.

4. A compound as claimed in any of claims 1 to 3 wherein X_1 represents aspartic acid, lysine, glutamic acid homolysine or a diaminoalkylic acid or derivatives thereof.

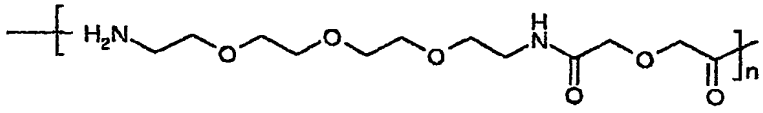
5. A compound as claimed in any of the previous claims wherein X_2, X_4 and X_6 independently represent a cysteine or homocysteine residue.

6. A compound as claimed in any of the previous claims wherein X_3 represents an arginine residue.

7. Compound as claimed in any of the previous claims wherein X_5 represents a phenylalanine, a tyrosine, a 3-iodo-tyrosine or a naphthylalanine residue.

8. A compound as claimed in any of the previous claims wherein X_7 is absent or comprises 1-10 units of a monodisperse PEG building block.

9. A compound as claimed in any of the previous claims wherein X_7 is absent or comprises 1-10 units of formula II

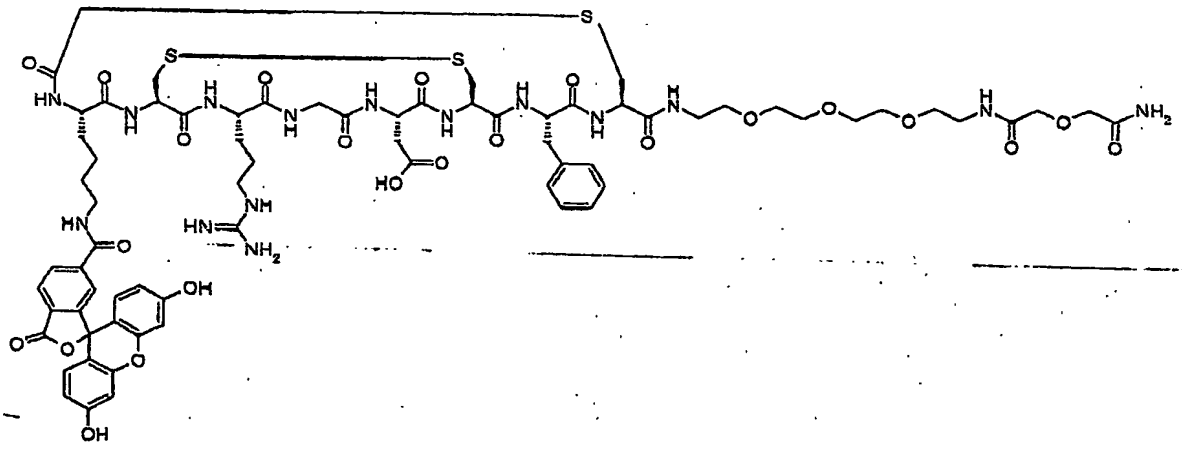


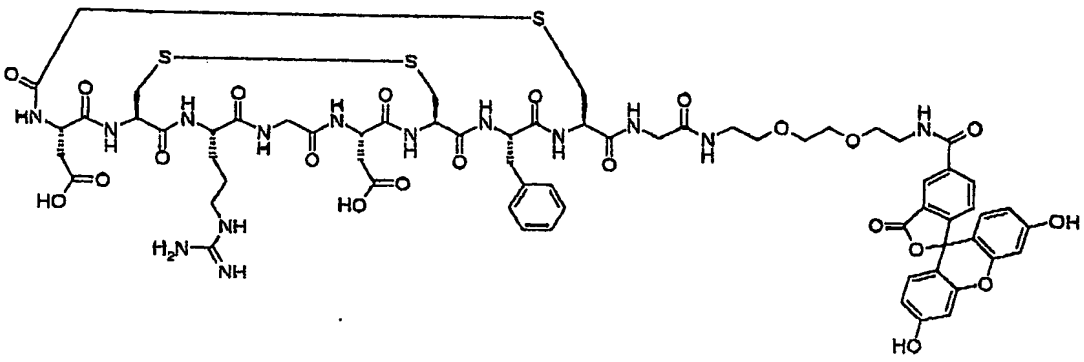
(II)

wherein the C-terminal comprises an amide or and acid function.

10. A compound as claimed in any of the previous claims wherein X_7 represent 1- 10 amino acid residues in combination with a PEG-like structure.

11. A compound as claimed in claim 1 defined by the following formulas:





12. A pharmaceutical composition comprising an effective amount of a compound of general formula (I) of any of the previous claims or a salt thereof, together with one or more pharmaceutically acceptable adjuvants, excipients or diluents for use in enhancing image contrast in in vivo imaging or for treatment of a disease.

13. Use of a compound as claimed in any one of claims 1 to 11 in the manufacture of an optical imaging contrast medium for use in a method of diagnosis involving administering said contrast medium to a human or animal body and generating an image of at least part of said body.

14. A method of generating images of a human or animal body by optical imaging involving administering a contrast agent to said body, and generating an image of at least a part of said body to which said contrast agent has distributed, characterized in that said contrast agent comprises a compound as claimed in any one of claims 1 to 11.

15. A method of generating enhanced images by optical imaging of a human or animal body previously administered with a contrast agent composition comprising a compound as claimed in any of claims 1 to 11, which method

comprises generating an image of at least part of said body.

16. A method of monitoring the effect of treatment of a human or animal body with a drug to combat a condition associated with angiogenesis, said method involving administering to said body a compound or composition as claimed in any one of claims 1 to 12 and detecting the uptake of said compound or composition by cell receptors, said administration and detection optionally but preferably being effected repeatedly, e.g. before, during and after treatment with said compound or composition.

Abstract

The invention relates to new peptide-based compounds and their use in diagnostic optical imaging techniques. More specifically the invention relates to the use of such peptide-based compounds as targeting vectors that bind to receptors associated with angiogenesis. The compounds are labeled with fluorescein and may be used as contrast agents in optical imaging in diagnosis of angiogenesis-related diseases.

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